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Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor

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Antidepressants increase adult hippocampal neurogenesis in animal models, but the underlying molecular mechanisms are unknown. In this study, we used human hippocampal progenitor cells to investigate the molecular pathways involved in the antidepressant-induced modulation of neurogenesis. Because our previous studies have shown that antidepressants regulate glucocorticoid receptor (GR) function, we specifically tested whether the GR may be involved in the effects of these drugs on neurogenesis. We found that treatment (for 3–10 days) with the antidepressant, sertraline, increased neuronal differentiation via a GR-dependent mechanism. Specifically, sertraline increased both immature, double-cortin (Dcx)-positive neuroblasts (+16%) and mature, microtubulin-associated protein-2 (MAP2)-positive neurons (+26%). This effect was abolished by the GR-antagonist, RU486. Interestingly, progenitor cell proliferation, as investigated by 5′-bromodeoxyuridine (BrdU) incorporation, was only increased when cells were co-treated with sertraline and the GR-agonist, dexamethasone, (+14%) an effect which was also abolished by RU486. Furthermore, the phosphodiesterase type 4 (PDE4)-inhibitor, rolipram, enhanced the effects of sertraline, whereas the protein kinase A (PKA)-inhibitor, H89, suppressed the effects of sertraline. Indeed, sertraline increased GR transactivation, modified GR phosphorylation and increased expression of the GR-regulated cyclin-dependent kinase-2 (CDK2) inhibitors, $p27^{Kip1}$ and $p57^{Kip2}$. In conclusion, our data suggest that the antidepressant, sertraline, increases human hippocampal neurogenesis via a GR-dependent mechanism that requires PKA signaling, GR phosphorylation and activation of a specific set of genes. Our data point toward an important role for the GR in the antidepressant-induced modulation of neurogenesis in humans.

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Introduction

Several studies have shown that antidepressants increase hippocampal neurogenesis in both animals and humans,[1–5] and whether this increase in neurogenesis is necessary to overcome behavioral deficits in animal models of depression is being intensely debated.[5–16] However, the molecular pathways underlying such effects have not been described to date. The effects of antidepressants on hippocampal neurogenesis have been demonstrated for different, chemically unrelated classes of antidepressants,[1] suggesting that a common molecular mechanism may underlie their neurogenic potential. Identifying such a mechanism would allow us to modulate neurogenesis, and thereby possibly counteract some of the neurobiological disturbances in depression.

Notably, recent studies have suggested that glucocorticoids are involved in the neurogenic action of antidepressants.[12,17] For example, one study has observed that antidepressants increase hippocampal neurogenesis only in mice that are co-treated with glucocorticoid hormones, but not in control mice.[12]

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The potential role of glucocorticoids in antidepressant-induced neurogenesis is also consistent with an extensive literature, some from our own group, which shows that antidepressants directly regulate the function of the glucocorticoid receptor (GR).[18–24] The GR is a ligand-activated nuclear transcription factor. Upon ligand binding, the GR translocates into the nucleus, binds to glucocorticoid response elements on the DNA and subsequently activates gene transcription (so called transactivation). Multiple kinases have been reported to differentially phosphorylate the GR at its serine residues, S203, S211 and S226, an effect which regulates GR nuclear translocation and GR-dependent gene transcription.[25–29] Studies by us and others have shown that antidepressants also induce GR nuclear translocation and transactivation, which is, at least in part, mediated by the cyclic AMP (cAMP)/protein kinase A (PKA) cascade.[21–24,30–33] Moreover, the intracellular availability of cAMP is tightly regulated by phosphodiesterase type 4 (PDE4); and, consistent with PKA-dependent regulation of the GR, the PDE4 inhibitor, rolipram, enhances GR activation by antidepressants.[30]

Considering this body of evidence, we hypothesized that antidepressants modulate adult hippocampal neurogenesis via a GR-dependent mechanism that requires PKA signaling and GR phosphorylation. To test this hypothesis in a clinically relevant model, we have used hippocampal progenitor cells from humans. Using this *in vitro* model, we have been able to separate the direct effects of antidepressants on progenitor cells from any potential indirect effects, which would be present in an *in vivo* model. Moreover, we have examined the differential effects of antidepressants (and glucocorticoid hormones) on progenitor cell proliferation and differentiation, as well as on GR phosphorylation, transactivation and subsequent changes in gene expression. In all our experiments, we have used the selective serotonin reuptake inhibitor, sertraline, which has recently been described as one of the most clinically effective antidepressants.[34] Confirmatory replication experiments have been conducted also using the tricyclic antidepressants, amitriptyline and clomipramine.

Materials and methods

Cell culture
The multipotent, human hippocampal progenitor cell line HPC03A/07 (provided by ReNeuron, Surrey, UK) was used for all experiments. Further information on this cell line and media components are available in the Supplementary Materials.

Differentiation assay
To assess changes in neuronal differentiation, HPC03A/07 cells were plated on black-sided 96-well plates (Nunclon, Roskilde, Denmark) at a density of 1.1×10^4 cells per well. HPC03A/07 cells were cultured in the presence of epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and 4-hydroxytamoxifen (4-OHT) for 72 h, and then washed and cultured in media without growth factors and 4-OHT for subsequent 7 days. Cells were treated with the antidepressant sertraline (1 μM), the GR-agonists, dexamethasone (1 μM) and cortisol (100 μM), the GR-antagonist RU486 (50 nM), the PDE4-inhibitor rolipram (100 nM) or the PKA-inhibitor H89 (50 nM). For these experiments, cells were treated during the initial proliferation phase and the subsequent differentiation phase (total treatment of 10 days), or only during the proliferation phase (72 h), or only during the differentiation phase (7 days). At the end of the total incubation time (10 days), cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature.

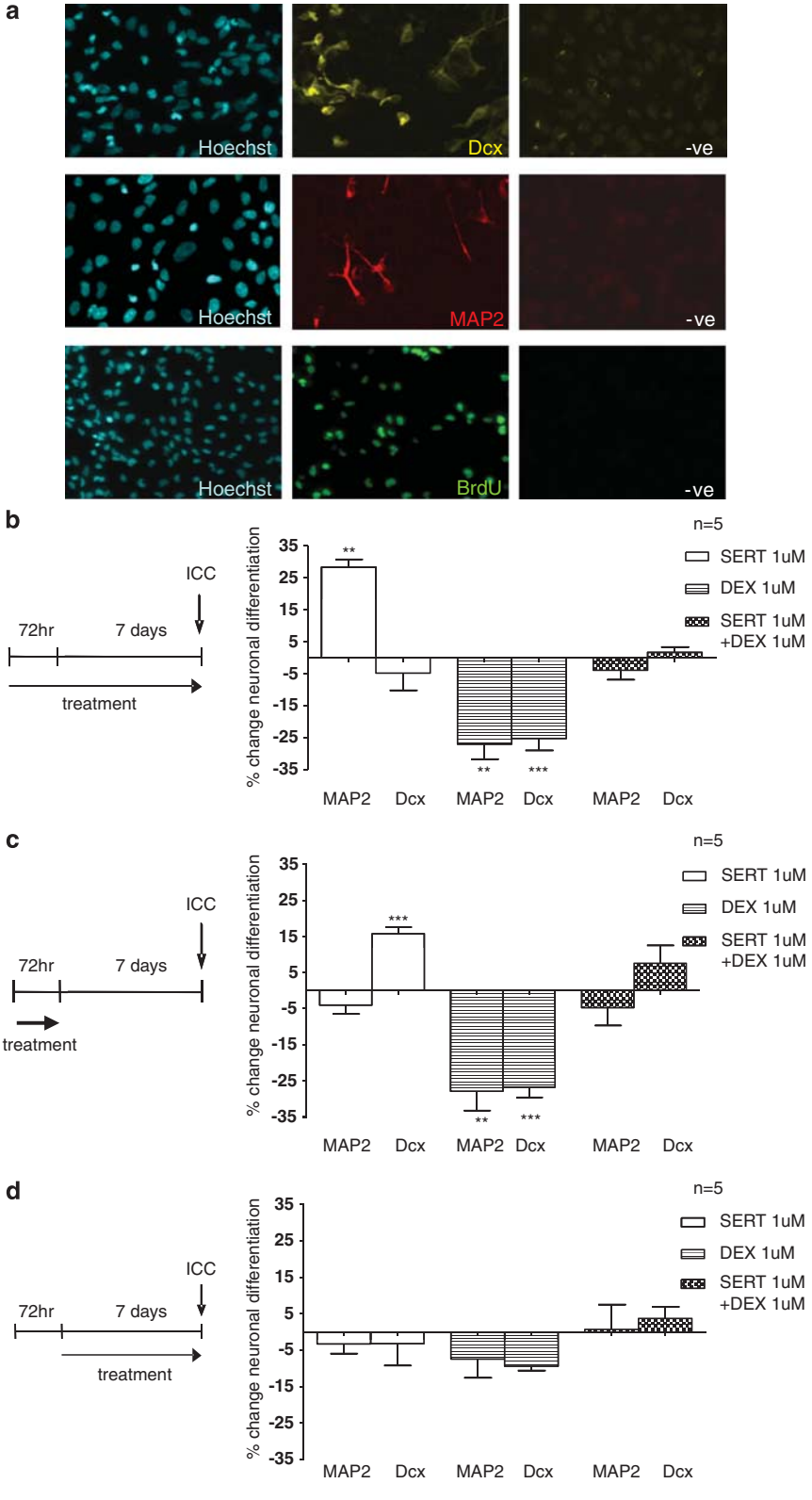
Proliferation assay
To assess progenitor cell proliferation, HPC03A/07 cells were plated as described above, and cultured for 72 h in the presence of growth factors and 4-OHT. The synthetic nucleotide 5′-bromodeoxyuridine (BrdU, 10 μM) was added to the culture media 4 h before the end of the incubation, and cells were fixed as described above.

Immunocytochemistry
Neuronal differentiation was assessed by doublecortin (Dcx), microtubulin-associated protein-2 (MAP2) and neuron-specific class III ß-tubulin (TuJ1) immunocytochemistry (Figure 1a, Supplementary Figure 2a). Specificity of the MAP2 antibody for mature neurons in our culture was confirmed by co-labeling experiments and Western Blotting (Supplementary Figure 11). Briefly, PFA-fixed cells were incubated in blocking solution (5% normal goat serum, Alpha Diagnostics, San Antonio, TX, USA) in phosphate-buffered saline containing 0.3% Triton-X for 2 h at room temperature, and with primary antibodies (rabbit anti-Dcx, 1:1000; mouse anti-MAP-2 (HM), 1:500, Abcam, Cambridge, UK; rabbit anti-TuJ1, 1:500, Sigma, St-Louis, MO, USA) at 4 °C over night. Cells were incubated sequentially in blocking solution for 30 min, secondary antibodies (Alexa 594 goat anti-rabbit, 1:1000 and Alexa 488 goat anti-mouse, 1:500, Invitrogen, Paisley, UK) for 1 h and Hoechst 3 3342 dye (0.01 mg ml^{-1}, Invitrogen) for 5 min at room temperature. The number of Dcx, MAP2 and TuJ1 positive cells over total Hoechst 33 342 positive cells was counted in an unbiased setup with an inverted microscope (IX70, Olympus, Hamburg, Germany) and ImageJ 1.41 software (http://rsbweb.nih.gov). To assess progenitor cell proliferation, BrdU-containing cells were incubated with hydrochloric acid (HCl, 2 N) for 15 min at room temperature, blocking solution for 60 min at room temperature, primary antibody (rat anti-BrdU, Serotec, Oxford, UK. 1:500) at 4 °C over night and secondary antibody (Alexa 488 goat anti-rat, 1:500, Invitrogen) for 2 h at room temperature. The number of BrdU-positive cells over total Hoechst 33 342 positive cells was determined as described above

(see Figure 1a). Negative controls were incubated with unspecific mouse IgGs (1:500, control for MAP-2), rabbit IgGs (1:500, control for Dcx and TuJ1) or rat IgGs (1:500, control for BrdU) in place of the specific primary antibody (see Figure 1a).

Western blot analysis of GR phosphorylation

To assess changes in GR phosphorylation, HPC03A/07 cells were treated for 1, 6 and 12 h. Cells were washed with ice cold phosphate-buffered saline containing phosphatase inhibitors (Pierce, Rockford,



IL, USA), scraped carefully from the flask and centrifuged in a pre-cooled centrifuge for 10 min at 3000 rpm at 4 °C. Cell pellets were resuspendend in protein extraction buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1% TritonX-100, 2 nM calyculin A (Cell Signaling, Danvers, MA, USA), 1 × protease and phosphatase inhibitors (Pierce)), and incubated for 15 min at 4 °C. Protein lysates were centrifuged for 15 min at 14 000 g at 4 °C. Protein concentrations were quantified using a bicinchoninic acid colorimetric assay system (Merck, Nottingham, UK) (see Supplementary Materials). Protein samples containing 50 µg of total protein were boiled for 10 min at 72 °C in 1 × NuPAGE LDS sample buffer (Invitrogen) and 1 × NuPAGE sample-reducing agent (Invitrogen), and subjected to reducing SDS-polyacrylamide gel electrophoresis on 10% NuPAGE Bis–Tris gels for 1 h at 200 V. Proteins were electrophoretically transferred to Immuno- Blot PVDF membranes (Bio-Rad laboratories, Hercules, CA, USA) at 110 V for 1.5 h at 4 °C. Transfer efficiency was controlled by Ponceau S staining and by pre-stained protein standards. Unspecific binding sites were blocked for 1 h in 5% bovine serum albumin in Tris-buffered saline, and membranes were immunoprobed with the polyclonal rabbit anti-P-S203 (1:10000) and anti-P-S226 (1:1000) antibodies (both from Dr Michael J Garabedian), anti-P-S211 antibody (1:500; abcam), anti-GR59 antibody (1:500, Fisher) and anti-beta-actin antibody (1:500, Biolegend, San Diego, CA, USA) in blocking solution at 4 °C over night. Membranes were washed with Tris-buffered saline containing 0.1% Tween-20, and incubated with a horseradish peroxidase-conjugated swine anti-rabbit secondary antibody (1:2000, DAKO, Glostrup, Denmark) in 5% non-fat dry milk in Tris-buffered saline, for 1 h at room temperature. Membranes were washed in Tris-buffered saline containing 0.1% Tween-20 and proteins were visualized with enhanced chemiluminescence detection system (GE Healthcare, UK). Data are expressed as fold change from the vehicle-treated control condition.

GR transactivation assay

To determine changes in GR transactivation, HPC03A/07 cells were treated with sertraline for 1, 6, 12, 24 and 72 h. Nuclear protein extracts were obtained using a commercially available nuclear extraction kit (Active Motif, Rixensart, Belgium). GR transactivation was analyzed using the enzyme-linked immunosorbent assay-based TransAM GR method (Active Motif), according to the manufacturer's instructions (see Supplementary Methods).

Gene expression analysis

RNA was isolated using RNeasy mini kit (Qiagen, Crawley, UK) and processed for gene expression analysis by quantitative real-time PCR using the SYBR® Green method (see Supplementary Methods). Each sample was assayed in duplicate and each target gene (*GR*, *GRα*, *p27^{Kip1}*, *p57^{Kip2}*, *p21^{Cip1}*, cyclin-dependent kinase2 (*CDK2*), cyclin A2 (*CCNA2*), cyclin D1 (*CCND1*), *p53*, human murine double minute2 (*HDM2*), FK506-binding protein 5 (*FKBP5*), serum/glucocorticoid-regulated kinase 1 (*SGK1*), forkhead box O1 (*FOXO1*), growth-arrest and DNA-damage-inducible ß (*GADD45B*), brain-derived neurotrophic factor (*BDNF*), *p11* and ß-*arrestin-2*) was normalized to the geometric mean of the three reference genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*) and beta-2-microglobulin (*B2M*). Pfaffl Method was used to determine relative target gene expression. Data are expressed as fold change from the vehicle-treated control condition. Primer sequences are available upon request.

Drugs

All drugs and reagents were purchased from Sigma–Aldrich (St Louis, MO, USA), unless otherwise stated. Growth factors EGF and bFGF were purchased from

Figure 1 Antidepressants induce differentiation and neuronal maturation of HPC03A/07 human hippocampal progenitor cells. Immunocytochemistry (ICC) for doublecortin (Dcx) and microtubulin-associated protein-2 (MAP2) was used to assess neuronal differentiation and maturation, respectively. 5′-bromodeoxyuridine (BrdU) incorporation and immunocytochemistry were used to assess progenitor cell proliferation (**a**). When HPC03A/07 cells were treated during the proliferation phase (72 h) and the subsequent differentiation phase (7 days), drug treatment had a significant effect on MAP2-positive neurons (one-way analysis of variance, $P<0.0001$, $F_{1,4}=62.22$, $R^2=0.9120$, $n=5$) and on Dcx-positive neuroblasts (one-way analysis of variance, $P=0.0007$, $F_{1,4}=13.28$, $R^2=0.6713$, $n=5$). Sertraline (SERT, 1 µM) increased the number of MAP2-positive neurons but did not alter the number of Dcx-positive neuroblasts, whereas dexamethasone (DEX, 1 µM) decreased both. No effect was observed upon co-treatment with SERT and DEX (**b**). When HPC03A/07 cells were treated only during the proliferation phase, drug treatment had a significant effect on MAP2-positive neurons (one-way analysis of variance, $P=0.0022$, $F_{1,4}=9.764$, $R^2=0.5824$, $n=5$) and on Dcx-positive neuroblasts (one-way analysis of variance, $P<0.0001$, $F_{1,4}=44.44$, $R^2=0.8724$, $n=5$). SERT increased the number of Dcx-positive neuroblasts, without an effect on MAP2-positive neurons (**c**). Treatment only during the differentiation phase did not have an effect on MAP2-positive neurons (one-way analysis of variance, $P=0.5699$, $F_{1,4}=0.6184$, $R^2=0.1709$, $n=5$) or on Dcx-positive neuroblasts (one-way analysis of variance, $P=0.2146$, $F_{1,4}=2.127$, $R^2=0.4596$, $n=5$) (**d**). Five independent experiments were conducted on five independent cultures ($n=5$), four wells were analyzed per treatment condition in each experiment and three random, non-overlapping pictures were analyzed for each well. All data are mean ± s.e.m. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the corresponding vehicle-treated control.

Peprotech (London, UK). Sertraline hydrochloride and rolipram were dissolved in 100% dimethyl sulfoxide dimethyl sulfoxide (DMSO); dexamethasone, hydrocortisone and RU486 were dissolved in 100% ethanol (EtOH); H89 dihydrochloride hydrate, amitriptyline hydrochloride and clomipramine hydrochloride were dissolved in sterile, deionized water. BrdU was dissolved in phosphate-buffered saline fresh before use.

Statistical analysis

Data are presented as mean ± s.e.m. All statistical analyses were performed with GraphPad Prism 4.03 (Graph Pad Inc, La Jolla, CA, USA) on independent biological replicates (indicated as n). One-way analysis of variance with Newman–Keuls *post-hoc* test was used for multiple comparisons among treatment groups. Student's *t*-test was used to compare means of two independent treatment groups. *P*-values < 0.05 were considered significant.

Results

Antidepressants and glucocorticoids regulate neuronal differentiation of human hippocampal progenitor cells

To investigate the effects of antidepressants on neuronal differentiation, we treated human hippocampal progenitor cells (HPC03A/07) with sertraline for 72 h during proliferation, and for subsequent 7 days of differentiation. Treatment with sertraline (1 μM) increased the number of MAP2-positive neurons (by 28%; Figure 1b, left white column), but did not significantly alter the number of Dcx-positive neuroblasts (Figure 1b, right white column). The specific GR-agonist dexamethasone (1 μM) decreased the number of MAP2-positive neurons and of Dcx-positive neuroblasts (by 27 and 25%, respectively; Figure 1b, striped columns). Treatment with cortisol induced the same effects of dexamethasone (Supplementary Figure 1a). Co-treatment of sertraline (1 μM) and dexamethasone (1 μM) abolished the reduction in MAP2 and Dcx-positive cells (Figure 1b, squared columns).

We also wanted to test whether treatment with sertraline during progenitor cell proliferation is sufficient to induce neuronal differentiation. We thus treated cells only during the proliferation phase, but not during the subsequent differentiation phase. Under these conditions, sertraline did not alter the number of MAP2-positive neurons (Figure 1c, left white column) but increased the number of Dcx-positive neuroblasts (by 16%; Figure 1c, right white column), indicating that sertraline initiates neuronal differentiation by an effect on progenitor cells, and only induces neuronal maturation if continuously present during neuronal differentiation (as shown in Figure 1b). Dexamethasone reduced the number of MAP2-positive neurons and of Dcx-positive neuroblasts (by 28 and 27%, respectively; Figure 1c, striped column), an effect that was abolished by sertraline co-treatment (Figure 1c, squared columns). Again, cortisol had the same effects of dexamethasone (Supplementary Figure 1b).

Interestingly, when we treated progenitor cells only during the differentiation phase (7 days), but not during the preceding proliferation phase (72 h), neither sertraline nor dexamethasone (or cortisol) altered the number of MAP2-positive neurons or of Dcx-positive neuroblasts (see Figure 1d and Supplementary Figure 1c). Taken together, these data indicate that the effects of antidepressants on proliferating progenitor cells are essential to induce neuronal differentiation into Dcx-positive neuroblasts, but not sufficient to promote their maturation into MAP2-positive neurons. Additional experiments in which cells were immunostained for the pan-neuronal marker TuJ1, confirmed these findings (see Supplementary Results and Supplementary Figure 2).

The effects of antidepressants on neurogenesis are dependent on the GR

We specifically wanted to test whether the GR is involved in the antidepressant-induced changes in neurogenesis. We, therefore, co-treated progenitor cells with sertraline and the GR-antagonist RU486 (50 nM), during both proliferation and differentiation. RU486 abolished the sertraline-induced increase in the number of MAP2-positive neurons (Figure 2a, white columns), thus confirming a role for the GR in the neurogenic action of sertraline. As expected, RU486 also eliminated the dexamethasone- (and the cortisol-) induced decrease in MAP2-positive neurons (Figure 2a, striped columns, Supplementary Figure 1). Furthermore, and consistent with the data described above, RU486 abolished the sertraline-induced increase in Dcx-positive neuroblasts, when cells were treated only during the proliferation phase but not during the subsequent differentiation phase (Figure 2b, white columns). Treatment with RU486 alone showed no effect on neuronal differentiation at the concentration used (50 nM) (data not shown). Taken together, these data support the notion that sertraline induces neuronal differentiation and promotes neuronal maturation via a GR-dependent effect.

The effects of antidepressants on neurogenesis are mediated by PKA signaling

Considering the role of PKA signaling in antidepressant drug action, we then wanted to examine whether the effects of sertraline on neurogenesis are mediated by the PDE4/PKA signaling cascade. Therefore, we co-treated cells with sertraline and the PKA-inhibitor, H89 (50 nM) or the PDE4-inhibitor, rolipram (100 nM), which enhances PKA activity by increasing cAMP levels. If treated during proliferation and differentiation, rolipram enhanced the effect of sertraline on MAP2-positive neurons (+43% vs sertraline alone), whereas H89 abolished it (Figure 3a, white columns). Interestingly, rolipram also counteracted the dexamethasone-induced reduction in MAP2-positive neurons, an effect that resembles the action of sertraline (Figure 3a, striped columns).

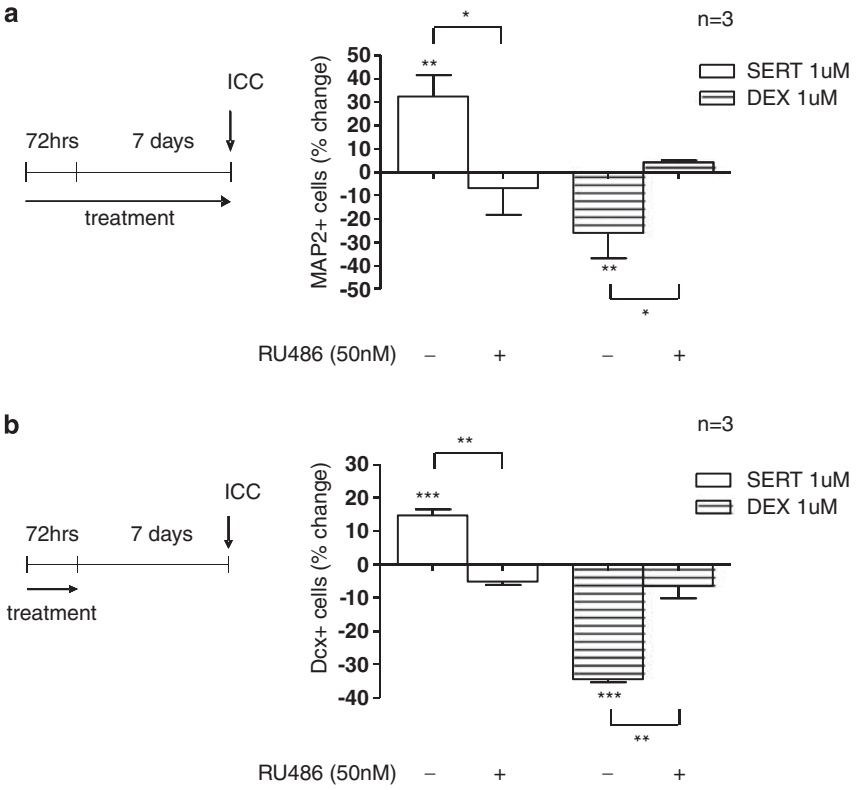


Figure 2 The effects of sertraline on neuronal differentiation are dependent on the glucocorticoid receptor (GR). The GR-antagonist RU486 (50 nM) abolished the effect of SERT (1 μM) and of DEX (1 μM) on the number of microtubulin-associated protein-2 (MAP2)-positive neurons (**a**) and on the number of doublecortin (Dcx)-positive neuroblasts (**b**). Three independent experiments were conducted on three independent cultures ($n = 3$), four wells were analyzed per treatment condition in each experiment, and three non-overlapping pictures were analyzed per well. All data are mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with SERT or DEX-treatment alone.

Likewise, if cells were treated only during the proliferation phase, rolipram enhanced the sertraline-induced increase in Dcx-positive neuroblasts (+105% vs sertraline alone) while H89 abolished this effect (Figure 3b, white columns). Rolipram also counteracted the dexamethasone-induced reduction in Dcx-positive neuroblasts, which again resembles the action of sertraline (Figure 3b, striped columns).

Antidepressants regulate human hippocampal progenitor cell proliferation

In all previous experiments, we examined the effects of sertraline on neuronal differentiation. In order to study the effects on cell proliferation, we treated progenitor cells with sertraline for 72 h, and incorporated BrdU (10 μM) during the last 4 h of incubation. Treatment with sertraline decreased the number of BrdU-positive cells (by 16%; Figure 4a, left white column), which supports the notion that progenitor cells stop proliferating in order to start to develop into neurons (as displayed in Figure 1). Again, as for the effects on neuronal differentiation, the effect of sertraline on progenitor proliferation was abolished by RU486 co-treatment (Figure 4a, right white column). Dexamethasone also reduced the number of BrdU-positive cells (by 20%), an effect that was again abolished by RU486 (Figure 4a, striped columns). A similar effect was observed using cortisol (Supplementary Figure 3a, black columns). Interestingly, proliferation was significantly increased only when progenitor cells were co-treated with sertraline and dexamethasone (+14%; Figure 4a, squared columns) (or sertraline and cortisol, Supplementary Figure 3a, squared columns). This effect was again abolished by RU486 (Figure 4a, squared columns). Treatment with RU486 alone showed no effect on proliferation at the concentration used (50 nM) (data not shown).

The same results were obtained using lower concentrations of BrdU (1 μM) (Supplementary Figure 4), using Ki67 immunocytochemistry (Supplementary Methods and Supplementary Figure 12), or using the tricyclic antidepressants, amitriptyline (1 μM) and clomipramine (1 μM) (Supplementary Figure 5). Cell death, as measured by propidium iodide live-staining, was not affected by dexamethasone, cortisol, sertraline, or dexamethasone and sertraline co-treatment in the concentrations used in this study (see Supplementary Materials and Supplementary Figure 10).

Finally, we wanted to investigate whether the effects of sertraline on cell proliferation also require PKA signaling. Indeed, rolipram enhanced the sertraline-induced reduction in cell proliferation (+37% vs sertraline alone), whereas H89 inhibited it (−41% vs sertraline alone; Figure 4b, white columns).

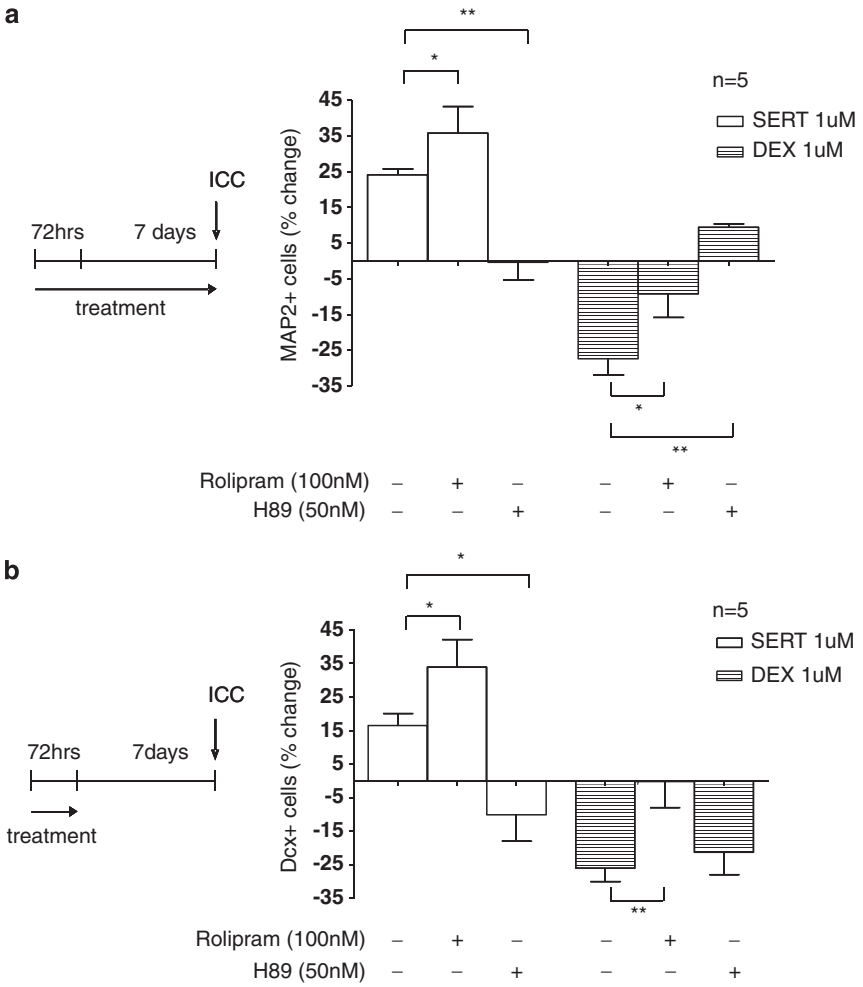


Figure 3 The effects of sertraline on neuronal differentiation are mediated by phosphodiesterase type 4 (PDE4)/protein kinase A (PKA) signaling. Rolipram (100 nM) increased the effect of SERT (1 μM) on the number of microtubulin-associated protein-2 (MAP2)-positive neurons, whereas H89 abolished it. Rolipram and H89 also counteracted the effect of DEX (1 μM) on MAP2-positive neurons (**a**). Rolipram increased the effect of SERT on the number of doublecortin (Dcx)-positive neuroblasts, whereas H89 abolished it. Rolipram also counteracted the effect of DEX (1 μM) on Dcx-positive neuroblasts (**b**). Five independent experiments were conducted on five independent cultures ($n = 5$), four wells were analyzed per treatment condition in each experiment and three random, non-overlapping pictures were analyzed for each well. All data are mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$ compared with SERT or DEX alone.

Moreover, co-treatment with rolipram and dexamethasone significantly increased proliferation (+17% vs vehicle; Figure 4b, striped columns), which again resembles the effect of sertraline and dexamethasone co-treatment. Finally, rolipram further increased proliferation upon co-treatment with sertraline and dexamethasone (+59% vs dexamethasone + sertraline), and this effect was abolished by H89 (Figure 4c, squared columns). These results support the notion that the effects of sertraline on cell proliferation, as those on neuronal differentiation, are mediated by PDE4/PKA signaling. It is important to note that rolipram did not change cell proliferation at the concentration used in these experiments (that is, 100 nM). However, higher concentrations of rolipram (10 μM) did indeed decrease cell proliferation via a GR-dependent effect (Supplementary Figure 6), further supporting a role for the interaction of PDE4/PKA and GR on neurogenesis.

Antidepressants modulate GR phosphorylation via PKA signaling

In the experiments above, we have demonstrated that sertraline, dexamethasone, and sertraline and dexamethasone co-treatment, have profoundly different effects on neurogenesis, but all these effects are mediated by the activation of the GR. One potential explanation for these results is differential regulation of GR phosphorylation by these three different treatment conditions. Therefore, we investigated GR phosphorylation at its serine residues S203, S211 and S226 at 1, 6 and 12 h of treatment. Our data indeed show that sertraline, dexamethasone, and sertraline

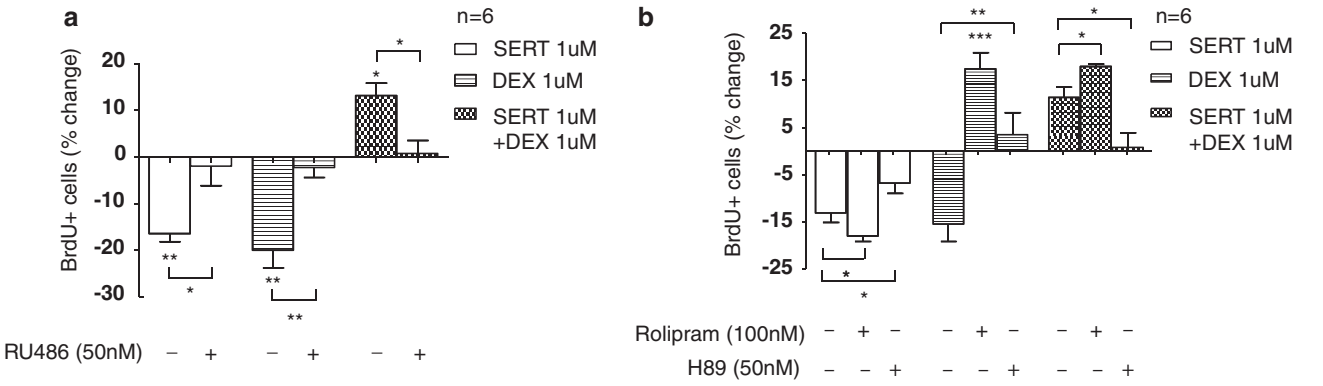


Figure 4 Effects of sertraline on progenitor cell proliferation. SERT (1 μM) and DEX (1 μM) decreased the number of 5′-bromodeoxyuridine (BrdU)-positive cells. These effects were abolished by the glucocorticoid receptor (GR)-antagonist RU486 (50 nM). Co-treatment of DEX and SERT increased the number of BrdU-positive cells. This effect was also abolished by RU486 ($*P<0.05$, $**P<0.01$) (**a**). Rolipram (100 nM) enhanced the effect of SERT (**b**, white columns), and of SERT and DEX co-treatment (**b**, squared columns), whereas H89 (50 nM) abolished both effects. The number of BrdU-positive cells was significantly increased when cells were co-treated with rolipram and DEX (**b**, striped columns). Six independent experiments were conducted on six independent cultures ($n=6$), four wells were analyzed per treatment condition in each experiment and three random, non-overlapping pictures were analyzed for each well. All data are mean ± s.e.m. $*P<0.05$, $**P<0.01$ and $***P<0.001$ compared with the respective vehicle or as indicated.

and dexamethasone co-treatment, activate distinct patterns of GR phosphorylation.

At the S203 residue, sertraline decreases GR phosphorylation after 1 h of treatment (0.5-fold compared with control; Figure 5a, first white column), whereas it increases phosphorylation at 6 and 12 h (both by 1.6-fold, white columns). In line with our data showing that the antidepressant effect on neurogenesis is dependent on PKA, inhibition of PKA by H89 abolishes all sertraline-induced changes in S203 phosphorylation of the GR (Figure 5a). In contrast, treatment with dexamethasone has the opposite effect: it induces S203 phosphorylation after 1 h of treatment (1.6-fold; Figure 5a, first striped column), and it decreases phosphorylation after 6 h (by 0.4-fold). Sertraline and dexamethasone co-treatment shows yet a third time-dependent phosphorylation profile: Although no changes in S203 phosphorylation are observed after 1 h (Figure 5a; first squared column), phosphorylation is increased at 6 and 12 h (by 1.5-fold and 1.7-fold, respectively; squared columns). These co-treatment effects are again blocked by H89. H89 alone did not show any effect at the concentration used (data not shown).

Similar differential effects of the three treatment conditions are present at the S211 phosphosite. Sertraline alone does not induce changes in S211 phosphorylation at any time point (Figure 5b; white columns). In contrast, dexamethasone strongly induces phosphorylation at S211 at 1, 6 and 12 h of treatment (by ninefold, twofold and threefold, respectively; striped columns). Interestingly, co-treatment with sertraline and dexamethasone induces hyperphosphorylation at S211 to levels higher than those induced by dexamethasone alone (12-fold at 1 h, 2.5-fold at 6 h, 4-fold at 12 h; squared columns). This hyperphosphorylation at S211 is also counteracted by H89, confirming again that the effect of sertraline on GR phosphorylation is indeed mediated by PKA signaling (Figure 5b).

No significant treatment effects were observed for phosphorylation at the S226 phosphosite (Supplementary Figure 9).

Antidepressants induce GR transactivation and enhance expression of the GR-dependent genes $p27^{Kip1}$ and $p57^{Kip2}$

To further elucidate the molecular mechanisms underlying the differential effects of sertraline, dexamethasone, and sertraline and dexamethasone co-treatment on neurogenesis, we compared the effects of these three treatments on gene expression of: the CDK2 inhibitors, $p27^{Kip1}$, $p57^{Kip2}$ and $p21^{Cip1}$; the cell cycle-regulating genes *p53*, *CCNA2*, *CCND1* and *HDM2*; and the glucocorticoid- and antidepressant-responsive genes, *FKBP5*, *SGK1*, *FOXO1*, *GADD45B*, *GR*, *GRα*, *p11* and ß-*arrestin-2*. These genes were chosen because they have all recently been implicated in antidepressant drug action and neurogenesis.[35–39,12,40–49] We conducted these experiments during cell proliferation, because, as described above, all effects (including those on differentiation) were initiated during the proliferation phase.

Gene expression analysis revealed that the three treatment conditions indeed activate distinct gene expression profiles. Details of the gene expression profiles and gene interactions for the three treatment conditions (at 6, 12, 24, 48 and 72 h) are described in Figure 5, Supplementary Results and Supplementary Figures 7 and 8. Here, we summarize the main findings.

Sertraline increased expression of the CDK2 inhibitors, $p27^{Kip1}$ and $p57^{Kip2}$ (Figures 5c and e), an effect that was particularly prominent after 12 h of treatment. Indeed, at this 12 h time point, sertraline

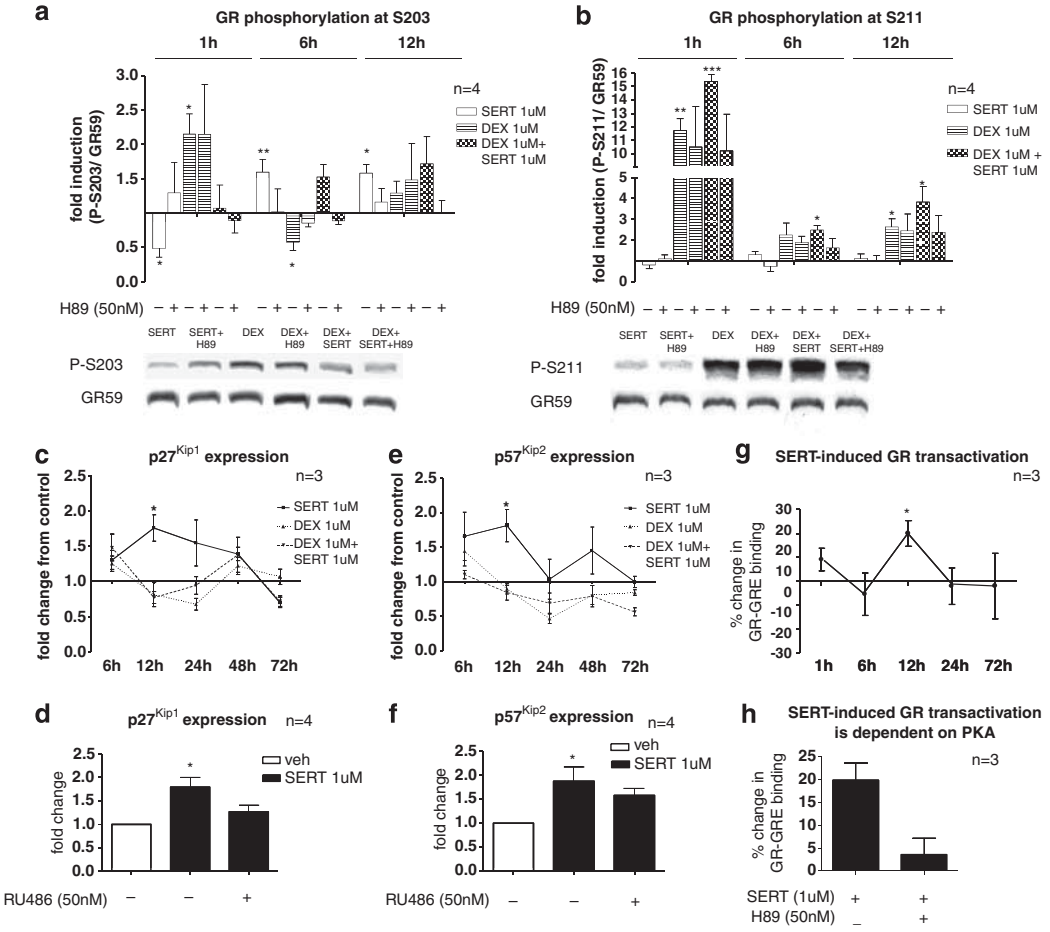


Figure 5 Sertraline regulates glucocorticoid receptor (GR) phosphorylation, GR-mediated expression of $p27^{Kip1}$ and $p57^{Kip2}$ and GR transactivation. During progenitor cell proliferation, SERT (1 μM) decreases GR phosphorylation at S203 after 1 h of treatment, but increases S203 phosphorylation after 6 h and 12 h (**a**, white columns). These effects are counteracted by H89. DEX increases S203 phosphorylation after 1 h and decreases S203 phosphorylation after 6 h (**a**, striped columns). DEX + SERT co-treatment does not change S203 phosphorylation at 1 h, but increases S203 phosphorylation at 6 h and 12 h (**a**, squared columns). These effects of DEX + SERT are also abolished by H89. SERT does not change GR phosphorylation at S211 (**b**, white columns). DEX increases S211 phosphorylation after 1, 6 and 12 h (**b**, striped columns). DEX + SERT co-treatment induces hyperphosphorylation at S211, which exceeds the phosphorylation induced by DEX alone, and this effect is also abolished by H89 (**b**, squared columns). Western blots for the GR-phosphoisoforms S203 and S211 are shown after 1 h of treatment (**a**,**b**). SERT increased the expression of $p27^{Kip1}$ (**c**) and $p57^{Kip2}$ (**e**). The increased expression of $p27^{Kip1}$ and $p57^{Kip2}$ after 12 h of sertraline treatment was abolished by RU486 (**d**,**f**). SERT increased GR transactivation after 12 h of treatment (**g**), an effect which was abolished by H89 (**h**). S203 and S211 phosphorylation are normalized to the expression of the unphosphorylated, total GR protein. Gene expression of $p27^{Kip1}$ and $p57^{Kip2}$ is normalized to the housekeeping genes ACTB, GAPDH and B2M. All data are mean ± s.e.m. $*P<0.05$, $**P<0.01$ and $***P<0.001$ compared with the respective vehicle.

also induced GR transactivation (+20%, Figure 5g; an effect, which was abolished by H89, Figure 5h) and reduced GR-expression (GRα mRNA by −30%, GR mRNA by −40%, GR protein by −50%; also blocked by H89; Supplementary Figure 7). The increased expression of $p27^{Kip1}$ and $p57^{Kip2}$ was also blocked by RU486 after 12 h of treatment (Figures 5d and f). Moreover, sertraline increased p11 and ß-arrestin-2 (Supplementary Figure 8l,m), and reduced GADD45B gene expression (Supplementary Figure 8j).

In contrast, dexamethasone reduced $p27^{Kip1}$ and $p57^{Kip2}$ (Figures 5c and e) and upregulated GADD45B, FKBP5, SGK1 and FOXO1 (Supplementary Figure 8g-j).

The only genes, which were regulated only by sertraline and dexamethasone co-treatment were the cell cycle-promoting genes, CCND1 and HDM2, which were both upregulated by this co-treatment (Supplementary Figure 8e,f).

Discussion

In this study, we identify for the first time that the antidepressant-induced changes in neurogenesis are dependent on the GR. Specifically, the selective

serotonin reuptake inhibitor antidepressant, sertraline, increases neuronal differentiation and promotes neuronal maturation of human hippocampal progenitor cells via a GR-dependent mechanism that is associated with GR phosphorylation via PKA signaling. Interestingly, this effect is only observed when sertraline is present during the proliferation phase, and it is accompanied by exit of cells from the cell cycle, as shown by reduced proliferation and increased GR-dependent expression of the CDK2 inhibitors, $p27^{Kip1}$ and $p57^{Kip2}$.

We and others have previously shown that antidepressants, including selective serotonin reuptake inhibitors, induce GR nuclear translocation,[50,21,22] modulate GR-dependent gene transcription,[21,24,50,30] and change GR expression in cell culture,[51–55,19] animals[19,52,56–61,36] and humans.[19,62–64] In this study, we identify for the first time a critical role for the GR in the antidepressant effects on neurogenesis: first, the sertraline-induced changes in neuronal differentiation and progenitor cell proliferation are abolished by the GR antagonist, RU486; and second, sertraline modulates GR phosphorylation, induces GR transactivation and changes the expression of GR-regulated genes relevant to neurogenesis (see below).

Surprisingly, sertraline (like amitriptyline and clomipramine) increases proliferation only in the presence of glucocorticoids (dexamethasone or cortisol), but not by itself. The strength of our *in vitro* model is that it separates the direct effects of antidepressants on progenitor cells from the indirect effects, which occur *in vivo*. Notably, increased hippocampal cell proliferation by antidepressant treatment in animals occurs always in the context of endogenous glucocorticoid production—which is not present in our *in vitro* system, unless when cells are co-treated. Indeed, some animal studies have reported that administration of exogenous glucocorticoids, or circadian fluctuations of endogenous glucocorticoids, are required for increased hippocampal cell proliferation upon antidepressant treatment.[12,17] Moreover, a study on human post-mortem brain tissue has found that antidepressants increase the number of neural progenitor cells in patients with major depression to levels above those present in controls, and depressed patients are generally characterized by elevated endogenous levels of glucocorticoids.[4] Taken together, our *in vitro* findings and the above mentioned *in vivo* studies indicate a pivotal role of the GR in the effects of antidepressants on hippocampal progenitor cells. It is important, however, to emphasize that our findings also demonstrate that the molecular processes that lead to increased neuronal differentiation are activated directly by antidepressants alone and do not require glucocorticoids. Our data suggest that such complex GR-dependent regulation of cell fate is the result of differential GR phosphorylation and GR-dependent gene expression by antidepressants, glucocorticoids and by antidepressant and glucocorticoid co-treatment. Indeed, different GR phosphoisoforms have been reported to selectively occupy promoters of different GR target genes, which may therefore explain the diverse GR-dependent effects on gene expression and neurogenesis that we observed in our study (see below).[26,65–68]

Further investigation of the molecular signaling mechanisms, which underlie the effect of antidepressants on neurogenesis showed that sertraline increases GR transactivation after 12 h of treatment. At this time-point, expression of the CDK2 inhibitors, $p27^{Kip1}$ and $p57^{Kip2}$, was strongly increased, and this increase was blocked by RU486. $p27^{Kip1}$ and $p57^{Kip2}$ are GR-target genes that promote cell cycle exit and increase neuronal differentiation in the developing rat brain.[39,38,69] Therefore, increased expression of these genes is consistent with our findings (discussed above), which show that sertraline decreases proliferation and initiates neuronal differentiation already during the proliferation phase. It is also of note that sertraline decreased GR expression (both at the mRNA and the protein level) at the same time point at which it induced GR transactivation. This is in line with reports from the literature in both cell culture and animal studies,[23,51,61] and probably results from the increased GR transactivation.[19,23,24] Finally, sertraline increased the expression of p11 and ß-arrestin-2, which have both recently been implicated in the antidepressant effects on neurogenesis *in vivo*.[12,40,42,43] Interestingly, the cell cycle-promoting genes, CCND1 and HDM2, were upregulated only by sertraline and dexamethasone co-treatment, the only condition which increases cell proliferation. In contrast, dexamethasone increased expression of the cell cycle-inhibiting genes, FOXO1 and GADD45B, which may explain the reduced cell proliferation and reduced neuronal differentiation with this treatment. See also Supplementary Discussion ('Gene interactions') and Supplementary Figure 13 for further discussion and a summary model.

Previous studies have proposed that antidepressants modulate GR-function by cAMP/PKA signaling.[30,31] Our data confirm and extend this model, by showing that the GR-dependent changes in neurogenesis upon sertraline treatment are mediated by PKA. Specifically, the PDE4-inhibitor, rolipram, which increases cAMP levels and thus leads to higher PKA activity, mimics and enhances the effects of sertraline on neuronal differentiation and progenitor proliferation. Accordingly, the PKA inhibitor, H89, inhibits the effects of sertraline. Furthermore, the sertraline-induced changes in GR phosphorylation and GR transactivation are also abolished by inhibition of PKA. One potential mechanism by which sertraline may cause this PKA activation is by liberating G-protein alpha(s) subunits from membrane-associated lipid rafts.[70–73] The subsequent cAMP production and PKA activation may then ultimately induce the GR phosphorylation, GR transactivation and GR-dependent gene transcription, which we observed in our study.[19,66,74,25,26] Interestingly, such a mechanism may be independent of monoamine reuptake systems, and could thus explain how neurogenesis is

modulated by antidepressants of different pharmacological classes[3] (and, in our experiments, by both the selective serotonin reuptake inhibitor, sertraline and the tricyclic antidepressants, amitriptyline and clomipramine).

In conclusion, our data suggest that antidepressants regulate differentiation and proliferation of human hippocampal progenitor cells by a GR-dependent mechanism that requires PKA signaling. This is accompanied by changes in GR phosphorylation and GR-dependent gene transcription, including increased expression of $p27^{Kip1}$ and $p57^{Kip2}$. Of note, our findings point toward a complex regulation of neurogenesis by antidepressants, with different GR-dependent mechanisms that lead to enhanced cell proliferation without changes in neuronal differentiation, or enhanced neuronal differentiation in the presence of decreased cell proliferation. It will be important for future studies to elucidate the impact of progenitor proliferation vs neuronal differentiation on depression and other mental illnesses. Moreover, modulation of GR phosphorylation and GR-dependent gene transcription may represent a future strategy of antidepressant drug treatment to overcome neurogenesis-related disturbances in depression.

Conflict of interest

Professor Jack Price acted as a consultant and received payment from ReNeuron Group within the last 2 years.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)